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Chapter

Comprehensive Account of Inoculation and Coinoculation in Soybean

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Abstract

This chapter elaborates dependency of leguminous plants on rhizobia to carry out dynamic process of nitrogen fixation. Soybean, an extensively grown leguminous crop with 30% share in world's vegetable oil, is taken into account to understand its symbiotic relationship with plant growth-promoting rhizobacteria (PGPRs). This chapter narrates colonization of PGPRs on soybean roots and single and mixed inoculation and coinoculation of certain strains of specialized bacteria with rhizobia. PGPRs' coinoculation seemed more effective than mono-inoculation and is discussed in Ref. to nodulation rate. Moreover, dynamic linear models for quantification of leguminous biological nitrogen fixation (BNF) are reviewed. This chapter further uncoils the relevance of foliar application to the release of phytohormones by PGPRs, resulting in situ biosynthesis of active metabolites in phyllosphere. Inoculation of phytohormones is compared to their exogenous application for nodule organogenesis. Finally, the influence of coinoculation on enhanced micronutrient bioavailability is relayed. The chapter is concluded with technical and economic aspects of coinoculation in soybean.

Keywords: legumes, nodulation, BNF, phytohormones, mixed inoculation

1. Introduction

Better plant growth is ensured by the balanced availability of essential nutrients in soil. Each nutrient has its own function and is required in different amount depending on the plant demand. Nitrogen (N), one of the most essential macronutrients, is routinely applied through chemical fertilizer as most field crops require large amounts of it. Nitrogen, the fifth most abundant element in the universe, was first discovered in 1772 by a Scottish physician, Daniel Rutherford. Due to its essentiality for survival of life on earth, it was called as "azote," meaning "without life," by Antoine Lavoisier about 200 years. Nitrogen is essential for the sustenance of life on this planet as it serves as building block for the synthesis of proteins. The inevitable role of N is well acknowledged in several biochemical processes such as cell division, growth promotion, and photosynthesis, as part of vitamins and carbohydrates and energy reactions in the plant body [1, 2]. Deficiency of N in plants is

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recognized by the symptom of delaying maturity of plant which leads to the late blooming. Deficiency symptoms also include chlorosis of leaves (light green or yellowing of leaves) and retarded plant growth. Due to high mobility of N, these deficiency symptoms first appear in older leaves of the plant [3].

The gaseous form of N is termed as dinitrogen (N_2) which accounts for 78% of the total gaseous content of the atmosphere. This form of N is unavailable for plants until it is fixed and converted into ammonium and nitrates, the forms in which plants can uptake N [4]. Soils contain both organic and inorganic N; however, organic form constitutes a major part of total soil N content. Plants, on the other hand, can use only specific inorganic forms of N like nitrate and ammonium. Like phosphorus (P) and carbon (C), N undergoes biogeochemical conversion from gaseous state to mineralized form in soil followed by its return to the atmosphere in the gaseous phase. The net concentration of N_2 per year was estimated to be 3×10^9 tons on global basis [5]. Nitrogen cycle is considered to be a biogeochemical cycle, where the N changes into different chemical forms and shifts to different ecological spheres of the earth. The fundamental components of N cycle are decomposers and N-fixing bacteria. Nitrogen cycle initiates with microbial fixation of N in the soil, where mineralization of N takes place by conversion of atmospheric or organic N into ammonium, a process known as ammonification. Further, ammonium is converted into nitrate by soil microbes and nitrifying bacteria, e.g., *Nitrobacter* and *Nitrosomonas* species. Denitrification is the ultimate step carried out by the denitrifying bacteria such as *Pseudomonas* and *Clostridium*, which decompose nitrate and convert it into N_2, thus returning N_2 back to the atmosphere.

2. Nitrogen fixation

The fixation of N involves conversion of N_2 into various nitrogenous compounds such as ammonium and nitrate, so that they may become more reactive and plant available.

2.1 Industrial N fixation

Industrial N fixation involves the Haber-Bosch process which is an energy-inefficient method for making nitrogen fertilizers:

$$N_2 + 3H_2 \xrightarrow{200°C,\quad 200\ atm} 2NH_3 \tag{1}$$

2.2 Natural N fixation

N fixation can be biological and nonbiological in natural environment.

2.2.1 Nonbiological N fixation (lightning)

In nonbiological fixation, a relatively small amount of N is fixed by a spontaneous reaction that occurs during lightning. It is estimated that about 10% of the world's supply of fixed N comes from lightning [6]. Lightning can be described as occurrence of a sudden electrostatic discharge during a thunderstorm. During lightning, atmospheric nitrogen reacts with oxygen to form nitric oxide (NO). In the presence of excessive O_2, nitric oxide oxidizes to nitrogen dioxide (NO_2). In the presence of water, NO_2 may react to form nitrous (HNO_2) and nitric acid (HNO_3) or may react with rainwater and oxygen to produce nitric acid. These acids find their way to reach the soil with rainwater, interaction with alkaline substrates

occurs, and hydrogen is released forming nitrate (NO_{3-}) and nitrite ions (NO_{2-}). The nitrate ions can be readily consumed by microbes and plants. However, soil microbes are not directly involved in this kind of N fixation. The chemical reactions involved in such N fixation are presented below:

$$N_2 + O_2 \xrightarrow{lighting} 2NO(Nitric\ oxide) \quad (2)$$

$$2\,NO + O_2 \xrightarrow{oxidation} 2\,NO_2(Nitrogen\ dioxide) \quad (3)$$

$$2\,NO_2 + H_2O \rightarrow HNO_2(Nitrous\ acid) + HNO_3(Nitric\ acid) \quad (4)$$

OR

$$4\,NO_2 + 2H_2O + O_2 \rightarrow 4HNO_3(Nitric\ acid) \quad (5)$$

$$HNO_3 \rightarrow H^+ + NO_{3-}(nitrate\ ions) \quad (6)$$

$$HNO_2 \rightarrow H^+ + NO_{2-}(nitrite\ ions) \quad (7)$$

2.2.2 Biological N fixation

Biological fixation of N_2 is carried out by N-fixing bacteria in soil. This fixation accounts for approximately 60% of fixed N in soil. Fixation of N_2 by microbes is termed as biological N fixation (BNF). Soil microbes are diazotrophs (bacteria and archaea) that contain enzyme nitrogenase, capable of converting N_2 into ammonium and nitrates, a process termed as nitrification. Common diazotrophs are rhizobia, blue-green algae (cyanobacteria), *Azotobacter*, *Frankia*, and green sulfur bacteria. Diazotrophs usually have a symbiotic relationship with leguminous family of plants. The major legumes are flowering plants like soybean, peanuts, clover, and lupines, tea plants like rooibos, and grasses such as alfalfa. The roots of legumes contain small protrusions called as nodules. These nodules are anchored by diazotrophs, providing anaerobic conditions for diazotrophs, further necessary for nitrogen fixation. Plants in turn use this fixed N for different functions. Upon death of the plants, this fixed N is released to the soil and acts as a nitrogen source for soil and non-leguminous plants. Nitrogen fixation is an energy-intensive process. One molecule of nitrogen gas breaks into its atoms and combines with hydrogen to form 2 molecules of ammonia at the expense of 16 molecules of ATP and a complex set of enzymes. Its reduction reaction can be written as:

$$N_2 + 3H_2 \xrightarrow{Energy} 2NH_3 \quad (8)$$

2.3 Classification of biological nitrogen fixation (BNF)

BNF can be classified into nonsymbiotic (free-living) and symbiotic (in association).

2.3.1 Nonsymbiotic biological nitrogen fixation

Microorganisms that fix atmospheric nitrogen independently are known as free-living diazotrophs. This type of fixation is carried out by free-living microorganisms. Examples of free-living organisms, which fix N, are cyanobacteria (blue-green algae, e.g., *Anabaena*, *Calothrix*, *Gloeothece*, and *Nostoc*), aerobic (*Azotobacter*, *Azospirillum*, Beijerinckia, *Derxia*), facultative (*Bacillus*, *Klebsiella*), and anaerobic

(non-photosynthetic such as *Clostridium* and *Methanococcus* and photosynthetic such as *Chromatium* and *Rhodospirillum*).

2.3.2 Symbiotic biological nitrogen fixation

Symbiotic nitrogen fixation, carried out by specialized soil bacteria as discussed above, is the good source of N for plants. In return, plants provide required nutrients and energy for bacterial growth. Upon the death of nitrogen-fixing bacteria, nitrogen is released to the environment, and some non-leguminous plants may benefit from that nitrogen. In leguminous plants, nitrogen-fixing bacteria colonize on plant roots forming nodules. Within these nodules, nitrogen fixation is carried out by the bacteria, and the end product, NH_3, produced is absorbed by the plant [7].

2.4 Legumes

Legumes belong to Fabaceae or Leguminosae family and are primarily grown for human consumption, as forage and silage for livestock, and act as a green manure for enhancing soil fertility. Some common legumes include alfalfa, soybeans, chick peas, pigeon peas, clovers, cow peas, kidney, lentils, mung beans, peanuts, peas, and vetches. These are native to tropical rain forests and dry forests in America and Africa [8]. Legumes consist of 750 genera and 19,000 species of herbs, shrubs, trees, and climbers.

Legume seeds (pulses or grain legumes) are the major part of human diet. Nutritionally, legume seeds are rich in protein contents as compared to cereal grains. The combined use of legumes and cereals may provide necessary dietary proteins. Legumes are also used as pasture and animal fodder in which soybeans are most commonly used. Legumes, as green manure, improve soil quality by adding nitrogen and organic matter. Legumes are used in crop rotation for the sustainable crop production. About 2500 species of Leguminosae produce root nodules.

2.5 Soybean

The soybean (*Glycine max* L.), commonly called soja bean or soya bean, is a legume species native to East Asia. It is enormously grown for edible seeds and oil extraction. The major countries involved in cultivation of soybean are the United States, Brazil, and Argentina. Soybean is the most economical source of vegetable protein around the world. It is also involved in the production of several chemical products. Many botanists believed that soybeans were first cultivated in central China earlier in 7000 BC and in the United States in 1804 [9]. Soybean appears to be an erect branching plant with length more than 2 m. It is a self-pollinated plant with adoption to various cultivable lands. This plant conveniently cultivate in fertile, well-drained, and sandy loam with relatively warm conditions. The vital source of N in legumes is nodulation prevailed by N-fixing bacteria. Soybean can fulfill 50–70% of its N demand from the air by establishing root nodules through adequate population of N-fixing bacteria.

2.6 Nodule formation

Nitrogen fixation in legumes starts with the formation of small, knob-like protuberances called nodules. The bacteria get all the necessary nutrients and energy from the plants. The roots of legumes release chemicals known as flavonoids to attract the bacteria [10]. In response to flavonoids, the soil bacteria produce nod

factors. Nod factors are signaling molecules which are sensed by the roots. As a result, a series of biochemical modifications lead to cell division in the root to create the nodule. Lectins, a sugar-binding protein in root hairs of legumes, are activated by nod factors. This helps in the recognition and attachment of rhizobial cells to the root hairs whose tips in turn become curved. The growing root hair curls around the bacteria in several attempts until one or more bacteria are enclosed. The enclosed bacteria colonize and eventually enter the developing nodule through infection thread. Infection thread is a structure extended through the root hair into the epidermis cell and then comes out of the root cortex. The bacteria are then surrounded by plant-derived membrane. Rhizobial multiplication starts in cortical cells which results in the formation of nodule on the surface. In side nodules, the bacterial cells continue multiplication and colonization until host cells are completely filled. After that bacterial cell becomes dormant bacteroids and starts floating in leghemoglobin. Leghemoglobin is a reddish pigment in cytoplasm of host cells which efficiently scavenges O_2 so that maintenance of the steady state of oxygen and stimulation of ATP production is possible. Plants provide shelter and organic compounds to the rhizobia, and in turn rhizobia provide fixed nitrogen to the plant. Among leguminous crops, soybean takes great consideration due to higher contribution of BNF. Normally, nodulation occurs after 4 weeks of plantation. The small nodules become visible after 1 week of the infection. The color of nodule appears white or gray when nitrogen fixation is insufficient, whereas color changes to pink or reddish as N_2 fixation progresses. This color change is attributed to the occurrence of leghemoglobin which is similar to blood hemoglobin that regulates the flow of oxygen to the rhizobia.

Perennial legumes such as alfalfa, clover, etc. develop nodule about half an inch capable of fixing N throughout the growing season. Annual legumes like beans, soybeans, and peanuts have short-lived nodule, round in shape with size of pea. These nodules are continuously replaced during the growing season. Annual legumes provide nourishment to developing seed instead of nodules; therefore, nodules cannot fix N anymore. The number of nodules varies per plant species, e.g., on average beans comprised of <100 nodules per plant, soybean can have several 100 nodules per plant, and peanut may have >1000 nodules per plant. Nodules on annual legumes, such as beans, peanuts, and soybeans, are short-lived and round in shape and can reach the size of a large pea and will be replaced constantly during the growing season. At the time of pod fill, nodules on annual legumes generally lose their ability to fix nitrogen because the plant feeds the developing seed rather than the nodule. Beans have less than 100 nodules per plant, soybeans will have several hundred per plant, and peanuts may have 1000 or more nodules on a well-developed plant.

Nodulation is regulated by both external and internal processes. Soil temperature, soil N mineral content, acidity of soils, and water scarcity can be categorized as external factors, whereas autoregulation and ethylene are the most influential internal factors. Autoregulation of nodule (AON) specifies the number of nodules per plant. Leaf tissue via chemical signal can sense the onset of nodulation and inhibit it in the developing root. Such chemicals are leucine-rich repeat (LRR) receptor kinases that are crucial for autoregulation of nodule formation. The mechanism for nodule formation is coded by *enod40* gene also called nodulin 40. Its expression leads to relocalization of nuclear proteins.

Microbes inhabiting soil can be termed as plant growth-promoting rhizobia (PGPR) due to their multifuntionality in symbiotic relationship with plant. PGPRs play role in plant nutrition by mineralizing nutrients in rhizosphere. PGPRs as indicated by name actively participate in phosphate solubilization and production of siderophore, phytohormones, and several enzymes. The biochemical

characteristics of PGPR, for instance, lipopolysaccharides (endotoxins), homoserine lactones (signaling molecules), acetoin (preventing over-acidification in cytoplasm), and flagella (locomotive and sensory organs) help plants to develop systematic resistance against pests and pathogens. The PGPRs enhance tolerance against extremity of environmental conditions such as drought, nutrient deficiency, and prevalence of organic (pesticides) and inorganic (heavy metals) toxicity. PGPR, therefore, are considered as biofertilizers for sustainable agricultural practices.

3. Inoculation and coinoculation of PGPRs

Soybean develops symbiotic relationship with a range of PGPRs to fix nitrogen (N) and improve plant growth [11–13]. Establishment of symbiotic relationship between roots of the host plant and symbiont is a two-step process. In first step, host tissue is infected with rhizobacteria and in second nodule formation occurs. Plant roots contribute in the symbiotic relationship by releasing flavonoids, while rhizobacterium produces nodulation factors. Rhizobacterium is entrapped in plant hairs' curls, and infection threads are formed at the root hair curls, permitting bacterial invasion of the root tissue. The process of nodulation is initiated just below the infected point. Rhizobacterium may be restricted to infection threads, but mostly, they are released into nodule cells where nitrogen fixation occurs.

3.1 Inoculation

Inoculation and coinoculation of PGPRs have become a popular research area in recent crop production. The interest in rhizosphere microbiology was developed due to the beneficial effects of some free-living strains of bacteria on plant growth and disease control and maintaining good soil health. Initial studies were focused on bacterial genera including *Pseudomonas*, *Rhizobium* spp. *Azotobacter*, *Bacillus*, and *Azospirillum* to enhance plant growth by fixing atmospheric nitrogen [14, 15]. However, later research was shifted to elucidate the role of PGPRs in promoting plant growth by mineralizing organic phosphorous, solubilizing inorganic soil phosphorous, modulating plant hormones, and rendering plant tolerance to adverse environmental conditions [11, 16]. This has triggered diversified application of PGPRs' inoculation and coinoculation in various field crops. The term "inoculation" may refer to "natural or deliberate application of certain beneficial strains of bacteria to plant seeds or soil to enhance plant growth." Inocula, the strain of bacteria used in inoculation and coinoculation, may be native or alien, with inherent or engineered ability to colonize plant roots and promote plant growth. Plant growth-promoting genera may include different strains of *Bacillus*, *Pseudomonas*, *Agrobacterium*, *Rhizobium*, *Mycobacterium*, etc. Bacterial inoculation has increased yield in many crops using *Azotobacter* and *Bacillus* strains. Steadily, the research focus has been shifted to *Azospirillum* from *Azotobacter* due to better crop yields reported with the later. Similarly, *Bacillus subtilis* and *Pseudomonas* spp. have been proven to be effective in controlling plant pathogens of soil origins. One major positive effect of inoculation is the solubilization of inorganic phosphate in the soil to make it plant available. Root exudates greatly influence the colonization of rhizobacteria. However, one major challenge in successful inoculation is the colonization of PGPRs in the rhizosphere where indigenous microbes may limit survival of the introduced bacteria. This has been addressed through introduction of antibiotic-resistant *rhizobacteria*. Besides these, soils are complex heterogeneous environments with great variations in particle size distribution, pH, organic matter

content, temperature, water, and availability of nutrients that may greatly influence inoculation success.

3.2 Coinoculation

To overcome some limitations of inoculation and increase PGPRs' efficiency, coinoculation is now commonplace in experimental and field trials. The objective is to increase the consistency and frequency of nodulation rate in various plant species. By definition, coinoculation is the combined application of PGPRs and other bacteria, bestowed with some specialized functions, to increase the nodulation rate, plant growth, and plant tolerance to adverse environmental conditions. For example, coinoculation of PGPRs with nitrogen-fixing bacteria has caused earlier nodulation and greater intensity, better uptake of nutrients and water, and improved plant growth [11]. In another study, coinoculation of soybean plants with strains of *Pseudomonas* and *Bacillus*, in combination with *Sinorhizobium meliloti*, has improved plant phosphorous uptake [16]. Moreover, *Azospirillum* has been used to increase the rhizobia-legume symbiotic relationship in soybean to improve its nutritive value [12]. For coinoculation, in vitro strain selection or genetically engineered strains are commonly used [17]. It is generally assumed that one rhizobacterium may be less effective in diverse environmental conditions. Thus, mixtures of various rhizobacterial species are promising in enhancing plant growth. But the coexistence of different bacterial strains under normal and adverse field conditions may be a challenge. Nowadays, coinoculation of PGPRs with mycorrhizal fungi is being practiced to promote growth in various plant species [13, 18–19]. Moreover, some studies have been focused on combining free-living bacteria, PGPRs, and mycorrhizal fungi [20].

3.3 Efficiency of coinoculation for enhancing nodulation rate

Inoculation and coinoculation of plants with single or multiple PGPRs may bring changes in the number of root hairs, nodule formation, root exudation, and release of phytohormones in addition to several physiological and metabolic changes. Generally, the potential of a specific PGPR strain to enhance nodulation rate can be best judged in a single experiment; however, consistent performance needs multiple field trials. The initial study on the role of PGPRs in enhancing nodulation rate was conducted on *Rhizobium trifolii*. The efficiency of coinoculation may also be dependent on the hormones and enzymes produced by PGPRs. For example, *Azospirillum* produces indole-acetic acid and pectinase which affect the development of symbiotic relationship and ultimately the nodulation efficiency [21]. Corporate research is focused on developing commercial inocula; however, several challenges need to be overcome before the product can make sense to the users. These include but are not limited to explaining exact mode of action of PGPRs under individual circumstances, persistency of performance over different ecological environments, and the optimization of the fermentation systems.

4. Phytohormones released by PGPRs

Rhizosphere is the soil adjacent to the growing roots of a plant. A strong interaction exists between the roots and soil. The microbial activity in the rhizosphere makes the interaction even stronger. The interaction between the plants and microbes can be symbiotic, nonsymbiotic, neutral, and parasitic. There are a number of microbes that are found in the rhizosphere; these include bacteria, fungi,

actinomycetes, protozoa, and algae. Among these the most common is the bacterial population. Plant growth-promoting rhizobacteria are the bacterial biomass that colonizes the plant roots in the rhizosphere [22]. PGPRs have been reported to play many important functions in plants; these include nitrogen fixation and uptake, tolerance under stress conditions, and production of certain phytohormones, i.e., plant growth regulators, siderophores (iron-binding protein compounds), volatile substances, and also certain enzymes, i.e., glucanase and chitinase to protect plants against disease [23, 24].

Phytohormones are produced in low concentration but have greater influence on the biochemical, physiological, and morphological functions of plants. They function as chemical messengers to transfer cellular activities in higher plants [25]. During the abiotic stress condition, these phytohormones play vital roles through communicating different transducing signals, which may control the external and internal stimuli [26]. Also some of the phytohormones are identified as stress hormones like abscisic acid (ABA). These phytohormones have a significant role in various plant processes. ABA besides facilitation during biotic and abiotic stress also is critical for maintaining seed dormancy, growth regulation, inhibiting germination, controlling the stomatal closure, and fruit abscission [27]. The plant growth regulators produced include auxin, gibberellic acid, cytokinins, and ethylene. Ahmad and Hasnain (2010) [28] have reported that *Bacillus* spp. producing auxin showed positive effect on the growth of potatoes. Earlier research work has revealed that PGPRs inoculation improved plant tolerance to stress condition due to enhanced production of growth regulators [29, 30].

4.1 Effect of synthetic PGRs on release of phytohormones

Plant growth regulators (PGRs) are synthetically available and are used in commercial agriculture extensively. Through various investigations, it has been found that application of growth regulators at pre-sowing stage to the seeds may enhance the nutrient reserves, tissue hydration, growth, and yield of crops [31]. Khan et al. (2018) [32] found synergistic effects of PGPRs and PGRs on different qualitative parameters of crops, i.e., chlorophyll, sugar, and protein contents. They concluded that application of PGRs to the plants inoculated with PGPRs helped plants under stress conditions. Also the amount of PGRs applied exogenously to the plants may be stored as reversible conjugates, and they also release phytohormones as required by the plants at different growth stages. Also these PGRs are found effective in transferring accumulates from source to the sink [33, 34].

Also some of the researchers have reported that the release of phytohormones may be enhanced several times by the applications of some suitable precursor of the plant hormones. These precursors are utilized by the rhizobacteria and converted into active phytohormones, and they are continuously used by the plants [35]. Among these precursors, L-methionine is an important precursor of ethylene (C_2H_4), a gaseous plant hormone that positively affects at almost all stages of growth and developmental processes [36]. Application of L-methionine to the rhizosphere enhanced the ethylene production and has shown significant increase in the growth and yield traits of soybean [36].

4.2 Effect of coinoculation on release of phytohormones

The bacterial population in the rhizosphere sometimes modifies the formation of nodules when they are coinoculated. The mechanism behind this process is that the coinoculation may directly enhance the growth and development of plant by the

increase in microbial biomass, extending the root system by release of phytohormones, solubilization of phosphate in the rhizosphere, etc. Moreover, development of roots provides additional sites for nodule formation [37, 38]. Indole-acetic acid (IAA) is an important metabolite of auxin group produced by the *Azospirillum brasilense* bacteria in the presence of tryptophan. Also the *A. brasilense* may produce the IAA in the absence of tryptophan under aerobic condition in the presence of NH_4 [39, 40].

Some PGPRs produce allelochemicals which are phytotoxic in nature. Production of these allelochemicals may adversely affect the soil health [41], by having negative effect on the enzymatic activity and plant functions, and may also hamper the nutrient availability to plants. The number of allelochemicals has been isolated from the bacterial strain present in the rhizosphere. It has been reported that a single strain of bacteria may produce a wide range of allelochemicals, e.g., *Streptomyces hygroscopicus* may produce nigericin and geldanamycin; these may be isolated and utilized as herbicides [42].

5. Influence of coinoculation on bioavailability of micronutrients

An exponential increase in the world's population will demand a higher production of food crops. By 2050, it is projected that the world's food demand will reach up to 3 billion tons. This high demand for food has been resulted in the excessive use of chemical fertilizer (nitrogen, phosphorus, potassium) in combination with advancements in technology to enhance the plant growth and production. Nitrogen is a vital nutrient in plant growth and productivity. Unfortunately, when a recommended dose of fertilizers is applied to crops for an average yield, less than 50% of applied nitrogen fertilizer is consumed by plants [43]. This low use efficiency of N causes the high fertilizer consumption and nitrate contamination of groundwater and soil which finally resulted in environmental degradation and health problems. Inoculation with microbes has been considered as an environmentally friendly alternative to minimize the use of synthetic nitrogen fertilizer without compromising the crop growth and yield [44, 45]. By biological nitrogen fixation, atmospheric nitrogen is converted to plant-utilizable forms, which is performed by microorganisms which convert the nitrogen to ammonia [46]. These microorganism generally is categorized into two groups: (i) nitrogen-fixing bacteria which generally includes the Rhizobiaceae family members and forms symbiotic associations with legume plants [47] and other non-leguminous plants and (ii) nonsymbiotic nitrogen-fixing bacteria (free-living, associative, and endophytic) such as *Cyanobacteria*, *Azotobacter*, *Gluconacetobacter diazotrophicus*, *Azocarus*, *Azospirillum*, etc. [48]. Rhizobia (including *Sinorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium*) are considered as symbiotic partners of legume plants and known by their role in the formation of N-fixing nodules in plant rhizosphere [49], while the nonsymbiotic N-fixing bacteria deliver only a small amount of fixed N which is required by the associated plant [50]. N-fixing PGPB strains and their effects on leguminous plants have been tabulated in **Table 1**.

Plant growth-promoting bacteria (PGPB) comprise a group of microorganisms that colonize the internal plant tissue and root surface and provide many benefits to host plants [51, 52]. These microorganisms can improve plant growth by contributing several mechanisms and processes including synthesis of hormones such as cytokinins, auxins [53], ethylene [54], gibberellins [55], and a variety of other molecules [56], biological control of pathogens [57, 58], and solubilization of phosphate [59]. Combinations of these mechanisms finally benefit the plant by

Bacterial strains	Plant	Effect	References
Azotobacter chroococcum	*Avena sativa* L.	Improved growth and yield	Devi et al. [70]
Azospirillum brasilense	Maize	Improved N use efficiency and improved yield	Morais et al. [71]
Bradyrhizobium spp. + *Azospirillum brasilense*	Soybean	Promoted growth and yield with N application	Hungria et al. [79]
Rhizobium	Chickpea	Promoted growth in combination with N application	Namvar et al. [80]
Bradyrhizobium, Azospirillum	Soybean	Significantly improved nodule biomass	Chibeba et al. [81]
Rhizobium sp. *BARIRGm901*	Soybean	Increased nodule weight and crop yield, improved the activity of nitrogenase enzyme and nitrogen assimilation	Alam et al. [82]
Diazotrophic bacteria	Rice	Increased grain yield	Araujo et al. [83]
Ochrobactrum ciceri Ca-34, Mesorhizobium ciceri TAL-1148	Chickpea	Improved nodule biomass and crop yield	Imran et al. [84]

Table 1.
N-fixing PGPB strains and their respective effect on leguminous plants.

improving growth [60, 61] and biological nitrogen fixation and increase the activity of nitrate reductase when growing as plant endophytes [62]. These bacteria also produce the siderophores and synthesize enzymes, antibiotics, or fungicidal compounds that protect the plants against phytopathogenic microorganisms [63, 64]. There are several factors such as agricultural practices, plant genotype, bacteria species, and strain that may affect the success of inoculation and plant response to these PGPB [65, 66]. Chickpea and *Rhizobium leguminosrum* subsp. *Cicero* associations, for instance, produce up to 176 kg/ha annually depending on environmental factors, cultivars, and bacterial strain [67]. *Azorhizobium caulinodans* is root- and stem-nodulating nitrogen-fixing bacterium which has been isolated from the stem nodules of *Sesbania rostrata (Bremek and Oberm.)* [68]. By endophytic colonization of non-legume roots, i.e., wheat, it can stimulate root growth and increase nitrogen content and yield [69]. Devi et al. [70] reported that the growth and yield of oats (*Avena sativa* L.) were increased due to the seed inoculation with *Azotobacter chroococcum* combined with the nitrogen fertilizer as compared to control and nitrogen fertilizer alone. Highest yield (239.02 quintal per hectare) was observed in *Azotobacter* seed inoculated +80 kg N as compared to control (111 q/ha) and nitrogen 80 kg/ha (205 q/ha) alone. In another study, Morais et al. (2016) [71] reported the effects of *Azospirillum brasilense* (inoculated in seed furrow) on maize growth and yield. Average maize grain productivity was observed to be 12.76 and 13.06 ton/ha when nitrogen is applied at the rate of 100 and 200 kg/ha, respectively. However, with the addition of seed furrow inoculation at the rate of 200 ml/ha, average grain productivity of maize was increased up to 13.21 and 14.0 ton/ha under the nitrogen application at the rate of 100 and 200 kg/ha, respectively. This PGPR improves the growth and yield by increasing the N and P content in plant, higher phosphate solubilization, ammonia, indole-acetic acid (IAA), and siderophore production [72]. Inoculation of seeds with *Rhizobium* increases the protein, chlorophyll content, nitrogen uptake, and growth parameter in legume crops [73, 74].

Now scientists have developed new microbial associations to avoid such negative interrelations and increase the effectiveness of biofertilizers. Consortia of PGPR with mycorrhizal algae [75] or fungi [51] can show a better performance as a result of cumulative or synergistic interactions between beneficial mechanisms of different microorganisms. Mycorrhiza is a symbiotic interaction between plants and soil fungi called as arbuscular mycorrhizal fungi (AMF). Both associates get benefits for this relationship by improving nutritional status, which reduces the needs of fertilizers for crops [76, 77]. Vesicular-arbuscular mycorrhizal fungi improved the availability of nitrogen and phosphorus to support the plant to survive in different environmental severe conditions [78].

6. Quantification of nodulation process by dynamic linear models

The symbiotic relationship between N_2-fixing bacteria and leguminous plants is a core factor in enhancing soybean crop yield around the world. The atmospheric nitrogen captured by these bacteria is enzymatically reduced to ammonia. This ammonia is assimilated by plant tissues in the form of nitrogenous compounds. Around 20–22 million tons of N is fixed by symbiotic rhizobia [85], while 17 million tons is removed or assimilated by aerial biomass of legumes [86]. The fixed N can serve as an inevitable resource of N depending on net N fixation in soil as compared to its removal or assimilation in aerial parts of legumes which is estimated to be 45–75% [87]. Nonetheless, the cropping systems with legumes have high crop yield as compared to non-legumes [88]. The fixation of N_2 can be maximized by sustainable and organic farming practices. However, legume specie, soil type and climatic conditions can also impact fixation rate of N_2 [89].The production of soybean as cash crop is evident in Brazil, Argentina, Russia, Ukraine, and the United States [90]. In Asia, North China and Japan chiefly cultivate soybean along with wheat [91].

Quantification of leguminous biological nitrogen fixation (BNF) can be beneficial for sustaining N demand and supply which can increase productivity and ability to combat environmental stresses. The techniques available for quantifying legume BNF are costly and protracted. Moreover, the data provided by such techniques are pertinent to limited time and space. Simulation of legume BNF is attainable by empirical and dynamic modeling. Empirical modeling is based on observation and experiment, while dynamic modeling is capable of representing a pattern or behavior over a time period. In case of legume BNF simulation, dynamic modeling can be desirable as it can correlate various environmental factors and legume growth status with N fixation. Broadly, legume BNF is discussed in relation to demand, uptake, and assimilation of N in biomass of root, nodule, and aerial parts of leguminous plants. Moreover, concentration of N accumulated in soil, along with soil's environmental parameters such as water content, N mineral concentration, internal substrate, C substrate and supply, and temperature are essential to quantify N fixation. Last but not the least growth rate of leguminous plant is a dynamic indicator in estimation of fixed N [92–95].

6.1 Estimation of N fixation by considering economic yield or aboveground biomass

During growing period, N fixation can be estimated by considering economic yield or dry matter of aerial biomass [96–98]. For this purpose, the equation can be:

$$N_{fix} = \alpha.DM.f_{leg}.N_{con}.\%Ndfa.(1 + R_{root}) \quad (9)$$

where DM represents dry matter of aerial biomass or yield, f_{leg} is proportion of legume crop in intercropping system, N_{con} is concentration of N assimilated in legume plant, and %Ndfa indicates proportion of N in crop which is derived from fixation of N_2, whereas R_{root} is a ratio of N fixed in belowground parts to the N fixed in aerial parts of legumes. α is a parameter which can have different definition depending on the researcher. For example, α can be used to represent correlation between decline in %Ndfa and high soil N content. In order to estimate total N input, α can be calculated as:

$$\alpha = 1 - \beta.N_{net.inorg} \tag{10}$$

where ß evaluates the responsiveness of legume for N fixation to already present mineral N (nitrate and ammonia) in the soil [98]. This method can directly estimate N fixation. Its parameter values can be taken both as estimated values from literature or measured values from on-site analysis. This method can work in the absence of previous data from past years. In these equations, environmental and weather conditions are not considered; therefore, this method can only be suitable for soils with similar properties and with exposure to moderate weather conditions. Moreover, the parameter values can be accustomed according to soil condition.

6.2 Linear empirical model

The empirical model can be used to explicit correlation between amount of N fixed in legumes and the total harvested part of legumes. In the case of intercropping system, fixed N in legumes can be correlated to the present fraction of legumes in the field. The equation is devised to calculate N fixation in kg N ha^{-1}, such as:

$$N_{fix} = c + d.Leg \tag{11}$$

where Leg denotes excess in harvested biomass (kg ha^{-1}) while c and d comprise the selected parameters.

The empirical model is based on statistical correlation with speculation of strong linear relationship between N fixation and variables. The applicability of this model is on wide variety of soils. This model requires adequate amount of data to constitute a correlation study and to determine the values for the selected parameter. The linear empirical model, however, does not account environmental conditions [99, 100].

6.3 Crop models as example of dynamic models

Leguminous N fixation in soybean was first simulated by Duffy et al. (1975) [101]. He estimated rate of N fixation by measuring root growth rate after specific days of planting. Crop models being dynamic in nature involve the potential impacts of soil environmental conditions for estimating N fixation. However, soil salinity, pH and availability of other nutrients are exempted in such models. Examples of crop models are Sinclair [102, 103], EPIC [104–106], Hurley Pasture model [107–110], Schwinning model [111, 112], CROPGRO [113–115, 93, 116], SOILN [117], APSIM [95, 118], Sousanna model [94] and STICS [119–121]. These crop models are applicable in varying environmental conditions; therefore, each model can have different versions for calculating N fixation. Thus, Liu et al. (2011) [122] devised a general equation for these crop models:

$$N_{fix} = N_{fixpot} f_T \, f_W f_N \, f_C \;\; f_{gro} \tag{12}$$

where N_{fixpot} indicates the potential rate of N to be fixed by legumes (g N fixed day^{-1}), f represents the influence function of environmental conditions, f_T is impact of soil temperature, f_W can be taken as impact of water deficiency or flooding in soil, f_N can estimate impact of availability of mineral N (nitrate and ammonia) in soil or N availability in root substrate, f_C represents effect of C concentration in root and aerial parts of legume plant, and f_{gro} is the effect of plant's growth stage on potential rate of N fixation. In the case of Environmental Policy Integrated Climate (EPIC) model and Simulateur mulTIdisciplinaire pour les Cultures Standard (STICS), the equation is generalized as:

$$N_{fix} = N_{fixpoint} f_T \;\; min \;\; \left(f_W, f_N\right) f_{gro} \tag{13}$$

where min indicates the minimum value that can be assumed between f_W and f_N. If applying STICS model, the limitation by anoxia is represented by extra function, i.e., f_a.

6.3.1 Potential N fixation

In dynamic models, the potential rate of N fixation is estimated on the basis of demand or uptake of N by legume plant or on the ability of root nodules to fix atmospheric N_2. In EPIC, the potential rate of N fixation is equal to the demand of N by legume plant [107]. The higher the demand of the N in legume plant, the higher will be the potential of N fixation. In contrast, according to Agricultural Production Systems siMulator (APSIM), the internal concentration of N in plant tissues governs the N demand of legume plant, which in turn defines the potential rate of N fixation in legumes. However, APSIM is applicable when plant has sufficient N concentration which can fulfill N demand of new tissues by uptake N from the soil [122]. N uptake is relatively passive and much preferable than N fixation; therefore, N fixation is only estimated when plant's demand for N is not fulfilled by N uptake [123]. Potential rate of N fixation, therefore, can be defined as difference between N demand and uptake [95, 118]. On the other hand, some researchers claim that the potential of N fixation is dependent on size and biomass of root and nodules, i.e., above- and underground biomass [124, 125]. However, estimation of N fixation using aboveground biomass is more convenient to handle than underground biomass [126].

6.3.2 Soil temperature

Soybean being plant of tropical and sub-tropical regions requires warm conditions for growing. The favorable temperature for soybean root zone ranges from 25–30°C [127]. Crop models such as Hurley Pasture model, CROPGRO, SOILN, and STICS estimated the effect of soil temperature on rate of N fixation by specifying certain temperature range. The generalized forms of equations are:

$$f_T = \begin{cases} 0 & \left(T < T_{min} \; or \; T > T_{max}\right) \\ \dfrac{T - T_{min}}{T_{optL} - T_{min}} & \left(T_{min} \le T \le T_{optL}\right) \\ 1 & \left(T_{optL} \le T \le T_{optH}\right) \\ \dfrac{T_{max} - T}{T_{max} - T_{optH}} & \left(T_{optH} < T < T_{max}\right) \end{cases} \tag{14}$$

where T represents soil temperature in °C, T_{min} is the minimum temperature below which N fixation can stop, T_{max} is the maximum temperature above which N fixation can stop to occur, and T_{optL} and T_{optH} indicate low and high values of optimal temperature range. In optimal condition, the optimum response to soil temperature becomes equal to the unit. Depending on location and legume species, the temperature range can vary in different models [109].

6.3.3 Soil water content

The excessive and deficient amount of soil water in the *Rhizobium* can negatively impact N fixation by the nodule. In STICS model, water deficit point is defined as segment of soil layers with water content above permanent wilting point [119]. Sinclair model, on the other hand, correlated transpirable water with nitrogenase activity of nodules [103, 104]. The nitrogenase activity is assessed by the reduction of acetylene which is used to explicit the proposed mechanism of BNF. The reduction in transpiration rate < 10% determines the transpirable water in soil, which in turn is stipulated by comparing the field capacity of soil and soil water content [102]:

$$f = -1 + \frac{2}{1 + e^{\left(-m * f_{TSW} + n\right)}} \tag{15}$$

where f_{TSW} represents the fraction of transpirable water in soil, whereas m and n are constants defining responsiveness of legumes for N fixation in low soil water content. APSIM, EPIC, and SOILN formulated linear function, which is expressed as:

$$f_{\omega} = \begin{cases} 0 & \left(W_f \le W_a\right) \\ \varphi_1 + \varphi_2 . W_f & \left(W_a < W_f < W_b\right) \\ 1 & \left(W_f \ge W_b\right) \end{cases} \tag{16}$$

where W_f is the ratio of relative availability of water content in soil at a given field capacity, W_a is the minimum value of water content below which N fixation cannot occur, ϕ_1 and ϕ_2 are the coefficients, and W_b is the threshold value of W_f above which N fixation is not impeded by water content of soil.

However, researchers with special focus on water stress conditions revealed that the top layer of soil around 30 cm is susceptible to dryness or wetness during dry spell or irrigation period. This can influence the access of water to root nodules [128]. Therefore, the presence of water within the roots is a more reliable factor in quantifying N fixation in limited water supply. Contrarily, in Hurley Pasture model, the chemical activity in the roots is assumed to control N fixation, wherein the chemical activity indirectly relies on probable water content in the root and temperature of soil [107]. So the effect of water is correlated with the thermal condition of soil such as:

$$f_W = e^{20 * \left[\frac{18 * \varphi rt}{8314 * (T_s + 273.15)}\right]} \tag{17}$$

where Φ_{rt}, probable water content in the root (J Kg^{-1}) and T_s is termed as thermal value of water content in soil (°C).

Excessive water can cause anoxic conditions in soil. In such condition, N fixation is assumed to be at zero in Sinclair model [103]. In anaerobic conditions, pore spaces become occupied with water; therefore, N fixation cannot occur.

6.3.4 Mineral N/internal substrate

The availability of N in the form of nitrates and ammonia is said to be mineral N in soil. In SOILN model, mineral N is incorporated for estimating N fixation in nodules such as:

$$f_N = \begin{cases} 1 - 0.0784 In\ N_s & (N_s \geq 1) \\ 1 & (N_{s<} 1) \end{cases} \tag{18}$$

where Ns is mineral N content of soil (mg N m^{-3}). The N uptake can be influenced by mineral N in soil; therefore, Schwinning model estimates potential of N fixation as:

$$f_N = \varepsilon \times \left(1.0 - f_{N_{up}}\right) = \varepsilon \times \left(1.0 - f_{max} \frac{1}{1 + K_N/N_s}\right) \tag{19}$$

where Ɛ is the efficiency of legume BNF, f_{max} is the maximum amount of N derived from the uptake of mineral N from soil, K_N indicates the concentration of nitrate in soil (g N m^{-2}) with N uptake reaching at half of its maximum rate, and Ns is the actual concentration of nitrate in soil (g N m^{-2}). In the given soil conditions, if nitrate concentration (N_{sNitra}) lies between 10 and 30 g (Nm^{-3}) within 30 cm topsoil layer, the EPIC model can be represented as:

$$f_N = \begin{cases} 1 & (N_{sNitra} \leq 10) \\ 1.5 - 0.05 N_{sNitra} & (10 < N_{sNitra} < 30) \end{cases} \tag{20}$$

In STICS model, high nitrate concentration in soil is assumed to inhibit nodulation progress which ultimately reduces potential rate of N fixation. If the concentration of nitrate in soil is higher than critical value, N_{fixpot} is set at baseline value; otherwise, N_{fixpot} is set at normal value [119]. In Hurley Pasture and Soussanna models, the plant substrate N concentration is included, such as:

$$f_N = \frac{1}{1 + N_{inter}/K_r} \tag{21}$$

where N_{inter} (g N g^{-1}r.wt) is assumed to be the N concentration in the root substrate (in Hurley Pasture model), or N concentration in plant substrate (in Soussanna model), and Kr is the coefficient for stating inhibition of N fixation at high nitrate concentration level in soil.

6.3.5 C in plant substrate or C supply

In plants, C is the source of energy for N fixation. Carbohydrate supports nodule biomass accumulation. The effect of C in estimating potential rate of N fixation is incorporated in Hurley Pasture and CROPGRO models such as:

$$f_C = \frac{1}{1 + K_C/C_r} \tag{22}$$

where Cr indicates concentration of C and Kc stands for Michaelis–Menten constant.

6.3.6 Plant growth stage

The impact of seasonal change on N fixation is incorporated in EPIC and STICS [106] such as:

$$f_{gro} = \begin{cases} 0 & \left(g < g_{min} \; or \; g > g_{max}\right) \\ \dfrac{g - g_{min}}{g_{optL} - g_{min}} & \left(g_{min} \le g \le g_{optL}\right) \\ 1 & \left(g_{optL} \le g \le g_{optH}\right) \\ \dfrac{g_{max} - g}{g_{max} - g_{optH}} & \left(g_{optH} < g < g_{max}\right) \end{cases} \tag{23}$$

where G_{min} is indicating the time period before which N fixation does not occur. This happens because of insufficient nodulation (expressed as % of total time period required for growing); g_{optL} is the initial time of growth and g_{optH} is the final time of growth. The time period between g_{optL} and g_{optH} represents N fixation by legumes, which is independent of growth stage. g_{max} is the growth time where N fixation stops due to deterioration of nodule.

The influence of symbiosis on metabolic fluxes and plant growth is quantified by a flux balance analysis. A genome-scale compartmentalized model for the clover (*Medicago truncatula*) as model plant has been devised by Pfau et al. (2018) [129]. The model predicted that nitrate uptake is significantly inhibited by the presence of ammonium in soil. When both nitrate and ammonium are available in soil, the uptake of ammonium is much favorable due to its integration into amino acids with fewer reductants and energy than nitrates.

The simulation of BNF by the abovementioned models included various biotic and abiotic factors to simulate and predict N fixation. Nodule biomass is more reliable to estimate N_{fixpot} than root and aerial biomass. C supply is considered to be the prominent factor in estimating N_{fixpot}. High concentration of nitrate in soil as mineral N can act as inhibitor for N fixation by nodules. Although empirical and dynamic models incorporated several factors such as soil temperature, water content, C, and other mineral contents, all the models lack information regarding the influence of soil pH and O_2 permeability. Therefore, adequate experimental work is required to cumulate the effect of such factors on biological fixation of N in legumes.

7. Technical and economic aspects

The impact of inoculation and coinoculation with elite strains such as *Azospirillum* species *(A. brasilense)* and *Bradyrhizobium* species (*B. japonicum*, *B. elkanii*, *and B. diazoefficiens*) has been extensively studied [130, 131]. Inoculation of *Azospirillum* spp. directly influences grain yield by improving N availability and its uptake. Moreover, this strain is helpful in the synthesis of phytohormones and developing pest resistance [132]. Crop yield is considered to be a primal factor for estimating profitability; therefore, increments in revenue are based on increments in grain yield [133, 134]. Coinoculation, regardless of cultivar, is reported to increase profitability by 14.4% as compared to non-inoculated treatments [134]. The economic evaluation of soybean plant is based on variables such as number of pods per plant, 100 grain weight, and yield. The data is usually quantified in kg ha^{-1} at wet basis [134].

The production of soybean crop can be estimated by total operating cost (TOC) method [135]. TOC is the sum of cost of fertilizers, heavy machinery, labor, pesticides, interests, etc. The major expenses are contributed by mechanization and fertilizers besides the cost of desiccation, control of weeds, pests, and pathogens. The inoculation of *Azospirillum brasilense* has increased the TOC, whereas the lowest TOC was reported with inoculation of *Bradyrhizobium* strain. However, the highest soybean yield was obtained with coinoculation of *A. brasilense*, leading to higher financial returns. Inoculation with *Bacillus* and *Pseudomonas* led to significant improvement in protein and nitrogen contents in grains in addition to high yield [136]. Similar results were reported when *Rhizobium* and *Pseudomonas fluorescens* improved yield and protein content when inoculated in beans [137].

In some studies, foliar inoculation of PGPR is found to be more effective than inoculation or coinoculation. For instance, foliar inoculation of *Azospirillum* in later stages of plant growth is correlated to high N content in developing grains [12]. This is because of the release of IAA by *Azospirillum*, which instigated nodulation in secondary nodules, thereby facilitating N fixation and its uptake in growing soybean plants. Likewise, foliar inoculation of *Azospirillum brasilense* at advanced growing stage of soy plant proved to be much more effective than its inoculation and coinoculation with *Bacillus japonicum* at sowing stage [12, 138]. However, foliar application at sowing stage is unable to produce any noticeable improvement in grain yield [12]. Moreover, coinoculation of *A. brasilense* and *B. japonicum* is reported to increase leghemoglobin by 39%, leading to high proportion of active nodules which in turn increased N fixation [139].

Organic and inorganic fertilizers such as NPK fertilizer and farmyard manure used along with PGPR, i.e., *Azotobacter* and *Trichoderma*, are reported to produce the highest biomass yield [140]. However, the inoculation of *Bradyrhizobium japonicum* on seeds increased grain yield of soybean by 8.4% (222 kg/ha), while its coinoculation with *A. brasilense* in furrow yielded 16.1% (427 kg/ha) without applying any external N source [141]. Similarly, Hungria et al. (2015) [79] coinoculated seeds of soybean with *Azospirillum* and *Bradyrhizobium* which resulted in high crop yield (388 kg/ha) without using any N fertilizer. The onset of earlier nodulation in soybean crop has been observed by the coinoculation of *Bradyrhizobium* and *Azospirillum* [81]. Moreover, these researchers claimed that the presence of *Azospirillum* after 18 days after emergence (DAE) facilitated plants to environmental stresses. Phosphorus as an essential nutrient for root growth is also necessary for rhizobia to convert N_2 into mineralized N [142]. Depending on the genotype of soybean, other nutrients like P can be influential in nodulation [143]. These researchers carried out coinoculation of rhizobia with arbuscular mycorrhizal fungi (AMF) in deep and shallow root genotypes of soybean. Regardless of soil N content, P was found to be a limiting factor in increasing nodulation, with low P colonization of AMF increased, whereas with high P, nodulation progressed in deep root soybean. Microbial inoculants are quite economical, making inoculation as a sustainable approach in soybean production [144]. Hence, the introduction of PGPR at appropriate stage of plant cycle can be a beneficial and reliable procedure for low-cost investment and sustainable agriculture.

8. Future prospects and conclusions

The reliance on N fixation is inevitable in spite of application of inorganic N fertilizer in huge amount (18 million tons/year) [86]. Legume plants being highly nodulated have high potential for N fixation which can be further facilitated by sustainable agricultural practices for high crop production. Inoculation and

coinoculation with different strains not only positively impact crop yield but also improve nutrient value of grains. PGPRs are natural source of plant growth hormones especially IAA, prompting nodule growth whether applied at sowing or in later stages of plant growth. In some cases, foliar inoculation was more effective for nitrogen and protein assimilation in soybeans than inoculation and coinoculation at sowing phase. Among PGPRs, certain strains of *Azospirillum* have a great potential of replacing inorganic sources of N, making inoculation a more economical approach toward sustainable agriculture.

The viability of PGPR inoculants is susceptible to rhizospheric conditions of soil, for which the compatibility studies are a compulsion [145]. When applied in the field, certain bacterial species (endophytes and rhizosphere-restricted bacteria) become VBNC, i.e., viable but not cultivable [63]. This might occur due to stress encounter by bacteria while colonizing host cell. The reason for VBNC is still unknown, but it is common to most rhizobial species. The research at molecular and genetic levels might solve this mystery. Soils with high mineral N content (ammonium and nitrate ions) are more prone to N reduction, as PGPR can readily consume it. Therefore, the viability of an applied farming approach can indicate the accessibility of organic N content in soil [98]. Moreover, the soils with common physicochemical features and exposure to similar climatic conditions may differ in net reduction of N content. This may be due to probable surface or drainage runoff of organic N during agricultural practices [146]. However, the estimation of soil N mass balance (input and output) requires long-term study which in turn will be helpful in the election of suitable cropping system. The use of economical viable PGPR inoculants along with efficient cropping systems can increase the probability of stable N retention in soils. In the case of developing countries, the lack of knowledge and relevant technological restrains demand an immediate implication of research (i.e., PGPR inoculation at sowing or spraying on leaves) in field conditions, thus providing cost reduction benefits to farmers and empowering local communities.

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